Short title

Molecular mechanism of photoperiod sensing

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Article Title

Photoperiod sensing of the circadian clock is controlled by ELF3 and GI

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One sentence summary

ELF3 and GI are essential for circadian clock mediated photoperiod sensing.

Author Contributions

M.U.A., S.J.D. and M.Q. conceived the project. M.U.A. and A.D. performed the experiments. M.U.A. wrote the article with contributions of all authors.

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Abstract

ELF3 and *GI* are two important components of the Arabidopsis circadian clock. They are not only essential for the oscillator function but are also pivotal in mediating light inputs to the oscillator. Lack of either results in a defective oscillator causing severely compromised output pathways, such as photoperiodic flowering and hypocotyl elongation. Although single loss of function mutants of *ELF3* and *GI* have been well-studied, their genetic interaction remains unclear. We generated an *elf3 gi* double mutant to study their genetic relationship in clock-controlled growth and phase transition phenotypes. We found that *ELF3* and *GI* repress growth during the night and the day, respectively. We also provide evidence that *ELF3*, for which so far only a growth inhibitory role has been reported, can also act as a growth promoter under certain conditions. Finally, circadian clock assays revealed that *ELF3* and *GI* are essential *Zeitnehmers* that enable the oscillator to synchronize the endogenous cellular mechanisms to external environmental signals. In their absence, the circadian oscillator fails to synchronize to the light-dark cycles even under diurnal conditions. Consequently, clock-mediated photoperiod-responsive growth and development is completely lost in plants lacking both genes, suggesting that ELF3 and GI together convey photoperiod sensing to the central oscillator. Since *ELF3* and *GI* are conserved across flowering plants and represent important breeding and domestication targets, our data highlight the possibility of developing photoperiod-insensitive crops by manipulating the combination of these two key genes.

Introduction:

Rotation of the earth around its axis results in rhythmic oscillations in light and temperature during a 24-hour day/night cycle. As a consequence of evolving under these predictable changes, organisms have developed internal timekeeping mechanisms known as the circadian clock that enables them to anticipate periodic changes in their surrounding environment (de Montaigu et al., 2010; Anwer and Davis, 2013). Circadian clocks consist of three pathways: inputs, core oscillators, and outputs. Input pathways deliver external cues (also known as *Zeitgeber*, German for time-givers), such as ambient light and temperature, to circadian oscillators. The timing information from the *Zeitgeber* is received by core-oscillator components known as *Zeitnehmer* (German for time-takers) that help to reset and synchronize the clock with the local environment (entrainment). Once entrained, the oscillators generate a ~24h rhythmicity that can be sustained for long periods; even in the absence of environmental cues (i.e., free-running conditions, such as constant light and temperature conditions) (Inoue et al., 2017; Oakenfull and Davis, 2017). After synchronizing with the external environment, oscillators link to various processes to rhythmically regulate the levels of genes, proteins, and metabolites. This allows organisms to anticipate and adapt to the changing environment, such as seasonal changes in day length (photoperiod). The circadian clock thereby regulates various output pathways including photosynthesis, growth, disease resistance, starch metabolism, and flowering time (Andres and Coupland, 2012; Shin et al., 2013; Müller et al., 2014).

The central part of the clock, the oscillators, are composed of transcriptional-translational feedback loops (Nohales and Kay, 2016; Ronald and Davis, 2017). The *Arabidopsis thaliana* (Arabidopsis) oscillator consists of three such loops: a morning loop, an evening loop and a central oscillator. The central oscillator is comprised of two partially redundant myb-like transcription factors CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), and a member of the PSEUDO-RESPONSE REGULATOR (PRR) family TIMING OF CAB EXPRESSION 1 (TOC1/PRR1). This is a dual negative feedback loop where respective morning and evening expression of *CCA1/LHY* and *TOC1* repress each other (Wang and Tobin, 1998; Alabadí et al., 2001; Huang et al., 2012). In the morning, the core-oscillator components CCA1/LHY activate *PRR7* and *PRR9,* which later repress *CCA1/LHY,* together constituting the morning loop (Zeilinger et al., 2006; Nakamichi et al., 2010; Kamioka et al., 2016). The evening expression of TOC1 represses *GIGENTEA (GI)*, which in turn activates *TOC1* and formulates the evening loop (Locke et al., 2006; Kim et al., 2007; Huang et al., 2012). Besides these three fundamental loops, a complex of three evening phased proteins (known as evening complex or EC), consisting of EARLY FLOWERING 4 (ELF4), ELF3 and LUX ARRYTHMO (LUX), have been identified as an essential part of the core oscillator (Nusinow et al., 2011; Herrero et al., 2012; Huang and Nusinow, 2016). The EC is connected to all three loops of the oscillator. By direct binding to their promoters, the EC represses the transcription of *PRR9* and GI (Helfer et al., 2011; Herrero et al., 2012; Mizuno et al., 2014; Ezer et al., 2017). A direct repression of *ELF3* by CCA1 connects the EC with the central oscillator (Lu et al., 2012; Kamioka et al., 2016).

ELF3 is one focus of this study and it encodes a multifunctional protein that regulates several physiological and developmental processes. Consistently, *elf3* null mutants display pleiotropic phenotypes such as long hypocotyl, accelerated flowering, elongated petioles, and arrhythmia under free-running conditions, suggesting that several important pathways are disrupted (Hicks et al., 2001; Kolmos et al., 2011; Herrero et al., 2012; Anwer et al., 2014; Box et al., 2014). In addition to its role as a member of the EC in the core oscillator, it functions as a *Zeitnehmer* in the light input pathway.

Therefore, plants lacking *ELF3* display severe light gating defects (McWatters et al., 2000). A physical interaction of ELF3 and PHYTOCHROME B (PhyB) establishes a direct link between the oscillator and photoreceptors (Liu et al., 2001; Kolmos et al., 2011). For the regulation of rhythmic growth, ELF3 mainly relies on the EC binding to the promoters of major growth regulators *PHYTOCHROME-INTERACTING FACTOR 4 (PIF4)* and *PIF5*, causing their transcriptional repression during the night (Nusinow et al., 2011; Raschke et al., 2015). However, ELF3 can also inhibit PIF4 by sequestering it from its targets (Nieto et al., 2014). Consistently, the lack of *PIF4/PIF5* repression in *elf3* mutants results in accelerated growth during the night (Nozue et al., 2007; Box et al., 2014). In addition to growth, *ELF3* controls flowering time by acting on the major floral activator FLOWERING LOCUS T (FT) via direct repression of *GI* (Mizuno et al., 2014; Ezer et al., 2017). Interestingly, ELF3 repression of *FT* does not require *CONSTANS (CO)* (Kim et al., 2005). Taken together, functional presence of *ELF3* is essential for both plant growth and development.

The second protein in the focus of this study is GI, a large, preferentially nuclear-localized protein with domains of unknown functions (Panigrahi and Mishra, 2015). The gene's transcription is controlled by the circadian clock. Furthermore, it is post-transcriptionally regulated by light and dark (Fowler et al., 1999; David et al., 2006). GI regulates diverse developmental and physiological pathways. The role of GI in the control of photoperiodic flowering is well documented. Here, GI acts as a major activator of *FT* expression, either by directly binding to its promoter or by inducing the expression of *CO (Fornara et al., 2009; Sawa and Kay, 2011)*. Moreover, GI physically interacts with both red and blue light photoreceptors PhyB and ZEITLUPE (ZTL), respectively, indicating a functional role also in photomorphogenesis (Kim et al., 2007; Yeom et al., 2014). Consistently, *gi* mutants are defective in proper light responses and display elongated hypocotyls under both red and blue lights (Huq et al., 2000; Martin-Tryon et al., 2007). Although the underlying molecular mechanism of hypocotyl growth regulation is not fully understood, it relies at least partially on PIF4, since the growth promoting effect of *gi* mutations was fully masked by the absence of *PIF4 (de Montaigu et al., 2014; Fornara et al., 2015)*. The EC subunit *ELF4* is epistatic to *GI* in regulating hypocotyl length, suggesting that the *GI* effect on *PIF4* is EC dependent (Kim et al., 2012). However, *ELF4* masking of *GI* is specific to growth regulation because in flowering time control the genetic hierarchy between these two is reversed. Here, *GI* is epistatic to *ELF4*. To make the interaction between these two players even more interesting, both are working additively or synergistically in the control of the circadian clock (Kim et al., 2012). GI plays a pivotal role in generating robust circadian rhythms under natural conditions in a way that daily rhythms of its expression respond to day length that depends on the latitude of origin of Arabidopsis accessions (de Montaigu and Coupland, 2017).

Interestingly, GI co-localizes with the EC components ELF4, ELF3 and LUX in nuclear bodies (Yu et al., 2008; Herrero et al., 2012), where it physically interacts with ELF4 and ELF3 (Yu et al., 2008; Kim et al., 2013). ELF4 regulates GI subcellular localization and modulates its DNA binding ability by sequestering it from the nucleosome(Kim et al., 2013). Further, GI and ELF4 have differentially dominant influences on circadian physiological outputs at dusk and dawn, respectively (Kim et al., 2012). The functional importance of ELF3-GI interaction is unknown. However, it is reported that ELF3 regulates diurnal protein accumulation of GI by facilitating its degradation during darkness by a CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) mediated proteasomal mechanism (Yu et al., 2008). Consistent with the finding that ELF3 binds to the *GI* promoter and represses its transcription (Mizuno et al., 2014), all components of the EC were found to bind the *GI* promoter in a CHIP-Seq experiment, demonstrating a direct relationship between *GI* and the EC (Ezer et al., 2017).

As mentioned above, the genetic hierarchy between *ELF4* and *GI* is relatively well understood (Kim et al., 2012). Based on the observations that mutations in EC components exhibit similar defects (Herrero et al., 2012), a conserved genetic relationship between GI and other EC components seems reasonable. On the other hand, the finding that ELF3 likely functions also independently of the EC (Nieto et al., 2014) opens the possibility for a different pattern of genetic interactions between *ELF3* and *GI*.

In this study, we provide genetic support for the biochemical evidence of an EC independent function of ELF3. We furthermore demonstrate that *ELF3* and *GI* are essential clock *Zeitnehmers* that are required to synchronize endogenous signals with the external environment. In their absence the circadian clock fails to respond to light signals, resulting in the breakdown of the photoperiod sensing mechanism. From an applied perspective, this interaction has the potential to generate photoperiod-independent crops, possibly allowing the cultivation of numerous day light sensitive species in currently non-permissive latitudes.

Results:

ELF3 and *GI* are essential for photoperiod responsive growth and development

ELF3 and *GI* are two important factors involved in photoperiod responsive flowering (Andres and Coupland, 2012; Lu et al., 2012). A previous report has suggested that under long days (LD, 16h light/8 h dark) *GI* is epistatic to *ELF3* (Chou and Yang, 1999). *GI* is also epistatic to *ELF4*, another component of EC, further suggesting that flowering time control of the EC acts through *GI (Kim et al., 2012)*. However, it is unclear whether the suggested genetic hierarchy between *ELF3* and *GI* is universally applicable under a range of photoperiods. To investigate the environmental sensitivity of these genetic interactions in detail, we generated an *elf3-4 gi-158* double mutant (hereafter designated as *elf3 gi*) and measured flowering time in comparison to the corresponding single mutants *elf3-4* (hereafter designated as *elf3*) and *gi-158* (hereafter designated as *gi*), and the Ws-2 wild type (WT) under long day (LD, 16h light/8 h dark), short day (SD, 8/16), and neutral day (ND, 12/12) photoperiods. Consistent with reported phenotypes of *elf3* and *g*i null mutants (Zagotta et al., 1996; Fowler et al., 1999; Lu et al., 2012), *gi* and *elf3* flowered later and earlier, respectively, than WT under all photoperiods tested (Figure 1A). Furthermore, similarly to WT, both single mutant alleles flowered earlier in longer photoperiods than in shorter photoperiods, therefore displaying an intact response to the length of the light period. Interestingly, such a photoperiodic response was completely lost in the *elf3 gi* double mutant, where flowering time was unaffected by the photoperiod (Figure 1A). Moreover, while under LD and ND flowering time of *elf3 gi* was similar to *gi*, it was similar to *elf3* under SD (Figure 1A). Thus, unlike *ELF4*, where *GI* is epistatic under both LD and SD (Kim et al., 2012), no clear genetic hierarchy was observed between *ELF3* and *GI*, suggesting independent roles in flowering-time control.

Since transition from the vegetative to the reproductive phase is only one of several developmental processes influenced by the photoperiod, we next sought to determine whether *elf3 gi* is also insensitive to photoperiod during the early growth phase. A classic phenotypic output for vegetative growth is elongation of the juvenile stem (hypocotyl), which, like flowering time, is also determined by the length of the light period. In WT, the length of the photoperiod is inversely proportional to the length of the hypocotyl. However, this relationship is not linear. Until a critical photoperiod (14-16 h light) is reached, the growth inhibitory effect of the increased photoperiod remains intact. After this time point, a further increase in the photoperiod has almost no effect on growth (Niwa et al., 2009). To investigate the role of *ELF3* and *GI* in photoperiod growth control, we measured hypocotyl length of WT, *elf3*, *gi,* and *elf3 gi* seedlings grown under a range of photoperiods, from 24 hours darkness (DD), with a gradual increase of 2 hour light periods, to 24 hours light (LL) (Figure 1B, S1A-B, Tables S1-S2). In confirmation of Niwa et al. (Niwa et al., 2009) , an intact response to photoperiod was observed in WT with plants responding to an increase in day length with a decrease in hypocotyl length until the 16h photoperiod. After 16h, no significant decrease in hypocotyl length was observed. Albeit with an overall longer hypocotyl, WT-like response to the changing photoperiod was also observed in *gi* (Figure 1B, S1A-B, Tables S1-S2). Interestingly, both *elf3* and *elf3 gi* did not display an intact photoperiod response of growth inhibition. Unlike WT, the repressive action of longer photoperiods continued even after 16h. Notably, the effect of light repression was discontinued after 20h photoperiod in *elf3*, whereas, in *elf3 gi* it continued until LL (Figure 1B, S1A-B, Tables S1-S2). Thus, our data indicate a previously not recognized additive function of *ELF3* and *GI* in photoperiod sensing, which only becomes visible in the absence of both genes.

The EC controls hypocotyl elongation by regulating the expression of *PIF4 (Nusinow et al., 2011)*. Under LD and SD, the length of the *elf4 gi* double mutant is similar to *elf4*, indicating that *ELF4* is epistatic to *GI (Kim et al., 2012)*. Since ELF3, like ELF4, is also a component of the EC, a similar genetic hierarchy could also be expected between *ELF3* and *GI*. If so, hypocotyl length of *elf3 gi* and *elf3* should be similar. However, we found that under both LD and SD *elf3 gi* was significantly longer than *elf3* (Figure 1B, Light periods 8 and 16), suggesting an additive function of *ELF3* and *GI*. Together, these data demonstrate that both *ELF3* and *GI* are essential for photoperiod sensing at both juvenile and adult stages of plant development.

ELF3 promotes growth under blue light

Previously, *ELF3* was reported solely as an inhibitor of growth under a range of light quantities and qualities (Zagotta et al., 1996; Reed et al., 2000; Doyle et al., 2002). In our light-period growth analysis, we observed that under LL *elf3* is significantly shorter than WT (Figure 1B, light period LL), suggesting that *ELF3* could act as a growth promoter under LL. To better understand the details of the *ELF3* growth promotion function, we first grew WT, *elf3*, *gi* and *elf3 gi* again under LL, LD, ND and SD photoperiods in white light. Consistent with Figure 1B, compared to WT *elf3* displayed longer hypocotyls under LD, ND and SD (Figure S1C-D). Under LL conditions, however, *elf3* hypocotyls were significantly shorter than WT (Figure S1C), confirming the initial observation from the experiment displayed in Figure 1B. To narrow down the light-spectrum, we next grew the seedlings under constant red or constant blue light, as well as under the corresponding monochromatic diurnal LD and SD conditions. Under red light, the results were similar to white light conditions under all photoperiods tested with single mutants including *elf3* being longer than WT and even longer double mutants (Figures 1C, S1E-F). While the same picture emerged for seedlings grown under blue light photoperiods that included a dark phase (Figures S1E-F), seedlings grown in constant blue light (BB) differed. Here, the *elf3* single mutant surprisingly displayed a significantly shorter hypocotyl compared to WT (Figure 1C). Although contradictory to the accepted understanding of being a general negative regulator of growth, these observations reveal a previously unknown growth promoting role of *ELF3* specifically under BB.

The growth inhibitory role of ELF3 in white light is known to be exerted at least in part via PIF4 (Nusinow et al., 2011). To better understand the *elf3* growth behavior under BB and to dissect the possibility of antagonistic action of ELF3 on *PIF4* under these conditions, we measured the expression of *PIF4* and its direct targets *IAA29* and *YUC8*. Interestingly, under BB, albeit a higher *PIF4* expression in *elf3*, the levels of its target genes were lower than in WT (Figure 1D). This indicates that in the absence of *ELF3* alone, PIF4 fails to fully induce the expression of its targets under BB, resulting in short hypocotyls. Provided that ELF3 affects the expression levels of these PIF4 target genes by acting on PIF4 itself suggests that under BB ELF3 exerts a growth promoting effect by positively influencing PIF4 activity. Also in agreement with their extended growth phenotypes under BB, *elf3 gi* double mutants express higher levels of the growth promoting PIF4 target genes (Figure 1D). A positive effect of ELF3 on PIF4 activity contradicts the previously described negative role of ELF3 in the regulation of PIF4 activity (Nieto et al., 2014). It is therefore possible that under BB ELF3 does not directly act on PIF4, but rather affects one of its many negative regulators (Quint et al., 2016).

***ELF3* and *GI* repress growth during night and day, respectively**

Under diurnal conditions, the elongation of hypocotyl is gated by the circadian clock, allowing maximum growth to occur at dawn under LD (Nozue et al., 2007). By repressing growth during the night, *ELF3*

functions as an important factor in clock gating. Consistently, *elf3* mutants have been reported to lose the normal gating response, resulting in maximum growth during the night (Nozue et al., 2007; Box et al., 2014). The role of *GI* in clock-controlled growth, however, remains largely unknown. The additive growth phenotype of *elf3 gi* (Figure 1B), reveals two possibilities: first, both *ELF3* and *GI* work cooperatively at a similar time of day. If so, the loss of both in the *elf3 gi* double mutant results in an increased growth at that particular time. Alternatively, both repress growth at a different time of the day-night cycle, resulting in an enhanced growth in *elf3 g*i at separate times. To dissect these possibilities, we measured growth rate of WT, *elf3*, *gi* and *elf3 gi* every hour for two days under LD using infrared imaging, which allowed growth monitoring also in darkness (Figure 2A-D). As reported previously (Nozue et al., 2007), maximum growth in WT was observed during the early morning at around ZT4 (Figure 2A). In *elf3*, the growth rate was overall increased with maximum elongation detected during the night (Figure 2B, Table S3), confirming the night-specific repressive function of ELF3 in elongation growth. The *gi* mutant displayed a broader growth peak during the afternoon with maximum growth observed at ZT8-10 (Figure 2C, Table S3). In *elf3 gi*, growth was pronounced during the night. However, in contrast to WT and both single mutants, growth rates did not peak at a specific time of day, but instead remained on a rather constant level. Compared to WT and the single mutants, the rate of elongation growth was increased during both day and night (Figure 2D, Table S3). Taken together, while we can confirm the previously described growth-repressive role of ELF3 during the night, our results reveal an unknown role of GI in repressing growth specifically during day times. For effective gating of clock-controlled growth, both ELF3 and GI are essential.

ELF3 and _GI_ work independently in the circadian clock

Since *ELF3* and *GI* are important components of the circadian clock (Mizoguchi et al., 2005; Anwer et al., 2014), we asked whether the photoperiod insensitivity of *elf3 gi*, as revealed by growth and flowering behavior shown above, could be attributed to a malfunctional oscillator. To investigate the interactive role of *ELF3* and *GI* in the clock, we monitored the expression of the *CCR2:LUC* reporter under constant light (LL) in WT, *elf3*, *gi* and *elf3 gi* plants that were previously entrained under LD, ND or SD (Figure 3). As expected for a functional oscillator, WT displayed a robust rhythm. In contrast, no rhythmic expression of the reporter was detected in *elf3* and *elf3 gi*. The *gi* mutant was also rhythmic albeit with lower amplitude (Figure 3A). Moreover, the levels of *CCR2:LUC* in *elf3 gi* were higher than the WT, and the single mutants *elf3 and gi* (Figure 3A-B), indicating an independent repressive function of *ELF3* and *GI* in the clock.

Using the same data, we next calculated the free-running period of the aforementioned lines. Irrespective of the photoperiod provided for entrainment, we found that the WT displayed a similar free-running period (Figure 3C). Compared to WT, an acceleration in clock speed was observed in *gi* (Figure 3C). Like WT, the photoperiod used during entrainment had no effect on *gi* periodicity (Figure 3C). Consistent with their arrhythmic phenotypes, no regular pattern of periodicity response to photoperiod was detected in *elf3* and *elf3 gi*. While *elf3* displayed an overall deceleration in circadian periodicity after all entrainment photoperiods, the *elf3 g*i response was more random, with a long and short period after LD and ND entrainment, respectively. After SD entrainment, the period of *elf3 gi* was similar to that of the WT (Figure 3C).

Next we assessed the precision of the oscillator by calculating the relative amplitude error (RAE). An RAE value of “0” represents a perfect rhythm, whereas an RAE of “1” typifies no rhythm(Anwer et al., 2014).

A general cutoff value of 0.5 is normally used to distinguish between a robust and a dysfunctional oscillator. As expected for a fully functional clock, the WT displayed a very low RAE after all entrainments (Figure 3D). The RAE measured for *gi* was significantly higher than the WT but lower than 0.5, suggesting a compromised but functional clock (Figure 3D). Consistent with their arrhythmic phenotype, the RAEs of *elf3* and *elf3 gi* were extremely high (RAE>0.6), indicating a dysfunctional oscillator. Collectively, a dysfunctional oscillator along with an increased *CCR2:LUC* expression in *elf3 gi* indicate an additive/synergistic role *ELF3* and *GI* in the clock.

Clock entrainment to light signals requires both a functional *ELF3* and *GI*

Several clock mutants that are arrhythmic under free-running conditions, display robust oscillations under diurnal conditions, suggesting that the oscillator is still capable of reacting to persistent environmental changes (Yamashino et al., 2008). The complete lack of response of the *elf3 gi* double mutant to photoperiod (Figure 1A-B and S1A-B), however, prompted us to think otherwise. Specifically, we hypothesized that the oscillator in *elf3 gi* might not be responsive to light signals even under diurnal conditions. To test this hypothesis, we monitored the expression of major central-oscillator genes *CCA1*, *TOC1*, *PRR9*, *GI* and *ELF3* under diurnal conditions (ND) (Figure 4A-E). In WT, the expression profiles of all these genes were consistent with previous data (Kolmos et al., 2011; Anwer et al., 2014), with *CCA1* and *PRR9* peaking in the morning, *TOC1* and *GI* peaking in the evening, whereas *ELF3* peaks in the night (Figure 4A-E). In *gi*, the expression of *TOC1* and *ELF3* was higher than the WT, whereas the levels of *PRR9* was lower than WT (Figure 4B-C,E), consistent with previous reports for *gi* null mutants (Fowler et al., 1999; Kim et al., 2012). No obvious difference in *CCA1* expression was detected in *gi* (Figure 4A). Also in agreement with published data, expression of *TOC1*, *PRR9* and *GI* in *elf3* was higher than in WT, while *CCA1* expression was lower (Hall et al., 2003; Kolmos et al., 2011; Anwer et al., 2014) (Figure 4A-D). Importantly, in both *elf3* and *gi*, albeit differences in expression levels, the overall shape of the expression patterns of all genes tested was similar to WT (Figure 4A-E). These data thus indicate an aberrant but functional oscillator in *elf3* and *gi* single mutants, which is capable of responding to environmental signals and generating robust rhythms under diurnal conditions. In *elf3 gi* double mutant, however, no detectable response to diurnal light signals were observed (Figure 4A-E). The expression profile of all clock genes tested were completely different from both single mutants and WT. Specifically, the overall expression of *PRR9* and *ELF3* was higher than the other genotypes tested. *CCA1* levels were almost non-detectable. The overall expression of *TOC1* was increased compared to WT and *gi* but decreased compared to *elf3*. The *GI* abundance was higher and lower in WT and *elf3,* respectively (Figure 4A-E). Most importantly, the characteristic peaks of expression of these genes, which were clearly detectable in WT, *elf3* and *gi,* were absent in *elf3 gi*. Most of the genes displayed a constant higher or lower expression, which was irresponsive to changes in the light during a diurnal cycle (Figure 4A-E). These data demonstrate that only in the absence of both *ELF3* and *GI,* the circadian oscillator is insensitive to persistent light-input cues. Thus, *ELF3* and *GI* are essential *Zeitnehmers* that are required for clock entrainment to external light cycles.

***ELF3* and *GI* are essential to establish endogenous and light signaling links**

Once entrained, the circadian clock regulates several key endogenous processes such as gene expression and ensures their precise synchronization with the external environment. This internal-external signaling synchronization is vital for several clock-controlled pathways such as flowering time and hypocotyl elongation. Since the oscillator in *elf3 gi* failed to establish a link with the external light signals, such a

synchronization could potentially be lost in *elf3 gi*, explaining its photoperiod-insensitive flowering and growth. This could be tested by monitoring the diurnal expression of key clock-regulated genes that are involved in photoperiod-responsive flowering and hypocotyl elongation as a proxy.

To investigate the functional ability of the *elf3 gi* oscillator to regulate its target genes, we first monitored the expression of the key flowering-time genes *GI*, *CO* and *FT* under ND (Figure 5A-C). Consistent with previous reports, we detected a rhythmic expression of *GI*, *CO* and *FT* in WT (Fowler et al., 1999; Sawa and Kay, 2011), with *GI* expressing during the day with the peak levels at ZT8, *CO* showing dual peaks, a smaller one at ZT8 and another one at ZT16-20. The maximum levels of *FT* were detected at dusk, at ZT12 (Figure 5A-C). Consistent with the late flowering phenotype of the *gi* null mutant, the expression of *CO* and *FT* was barely detectable in *gi (*Figure 5B,C). In *elf3,* the expression of *GI* was higher at almost all time points (Figure 5A), consistent with the direct repression of *GI* by ELF3 (Mizuno et al., 2014; Ezer et al., 2017). The expression of *CO* was higher during the early day and again during the night, whereas *FT* expression was only elevated during the day at ZT4 (Figure 5A-C). The expression pattern of *CO* and *FT* in *elf3 gi* was similar to *gi*. Notably, no diurnal peak of expression was observed in the *elf3 gi* double mutant for any of the genes tested, with the overall expression hardly fluctuating over the entire diurnal cycle (Figure 5A-C).

We further validated these results by monitoring the expression of the major growth promoter *PIF4* under ND (Figure 5D). Consistent with their long hypocotyls, an overall higher expression of *PIF4* was observed in *elf3* and *gi* (Figure 5D). Furthermore, in *gi, PIF4* followed a similar clock-regulated diurnal pattern as that of WT, albeit with marginally but consistently higher levels (Figure 5D). *PIF4* expression in *elf3* also followed a diurnal pattern. However, it showed a characteristic light regulated profile, with a gradual decrease in expression during the light period and a gradual increase during the dark period (Figure 5D). Interestingly, the *PIF4* expression in the *elf3 gi* double mutant was completely different from the diurnal patterns in WT and single mutants. Compared to WT, the level of *PIF4* was higher in *elf3 gi* at almost all time points, explaining for example its extreme growth phenotype shown in Figure 2. Further, *elf3 gi* displayed neither the clock regulated *PIF4* profile as observed for *gi*, nor the light regulated expression as observed in *elf3* (Figure 5D). A closer examination revealed that the *PIF4* levels remained almost similar throughout the diurnal cycle with the exception of ZT16 where expression levels were increased in comparison to other time points (Figure 5D). Collectively, these data demonstrate that both *ELF3* and *GI* are required for clock entrainment and thereby for the generation of rhythmic endogenous processes synchronized with the external signals.

Discussion:

The circadian clock is an important time keeping mechanism that synchronizes the internal cellular mechanism to the external environment. Light is the primary cue that provides timing information to the clock (Inoue et al., 2017; Oakenfull and Davis, 2017). While light sensing by the photoreceptors is well understood, it remains unclear how this information is perceived by the central oscillator. Here, we show that clock components *ELF3* and *GI* are essential to perceive light input into the clock and thereby for the measurement of the photoperiod. Absence of these components results in a dysfunctional oscillator, even under diurnal conditions, failing to regulate photoperiod-responsive growth and development.

Single loss of function mutants of individual EC components exhibit similar clock, hypocotyl and flowering time phenotypes, indicating that they work cooperatively (Nusinow et al., 2011; Herrero et al., 2012). Recent biochemical data has suggested that ELF3 can also function independently of the EC (Nieto et al., 2014). However, conclusive genetic evidence supporting the biochemical data is lacking. Previous data reported a clear genetic hierarchy between *ELF4* and *GI* with *ELF4* being epistatic to *GI* in control of hypocotyl elongation. *Vice versa*, *GI* is epistatic to *ELF4* in flowering time regulation (Kim et al., 2012). In our study, we did not observe such genetic relationships for *ELF3* and *GI*. Taking into account that ELF3 and ELF4 function together in the EC (Nusinow et al., 2011; Herrero et al., 2012), this is somewhat surprising, supporting the proposed EC independent function for *ELF3 (Nieto et al., 2014)*. The phenotypes we observed in single and double mutants for hypocotyl elongation suggest an additive function of *ELF3* and *GI* in controlling elongation growth (Figure 1B, S1A-F), whereas in flowering time regulation *ELF3* and *GI* were epistatic to each other under SD and LD, respectively. In circadian clock control, *elf3 gi* displayed similar additive/synergistic phenotypes (Figure 3A-B) as reported for *ELF4* and *GI*. Collectively, in agreement with the biochemical data, our genetic analyses demonstrate that *ELF3* function is not solely dependent on the EC.

ELF3 has been established as a repressor of growth that mainly works by acting on PIF4 (Nusinow et al., 2011; Nieto et al., 2014). Under diurnal conditions, the role of ELF3 as a growth inhibitor is undisputed. However, under constant light, contradictory phenotypes of *elf3* mutants were reported. Under LL, *elf3* mutants displayed either similarly long or slightly longer hypocotyls (Liu et al., 2001; Kim et al., 2005) compared to WT (Doyle et al., 2002; Park et al., 2017). Consistent with previous data (Kim et al., 2005; Kolmos et al., 2011; Nusinow et al., 2011; Lu et al., 2012; Anwer et al., 2014; Box et al., 2014; Raschke et al., 2015), under a range of photoperiods and light spectra, we consistently observed an elongated hypocotyl of *elf3* (Figure 1B, S1A-F). However, under LL, *elf3* was significantly shorter than WT (Figure 1B, S1C). Further experiments under different light spectra revealed that the growth promoting function of *ELF3* was photoreceptor dependent. Specifically, the *elf3* was shorter than WT under BB (Figure 1C). Interestingly, under these conditions *PIF4* levels were still increased in *elf3*, but it failed to induce the expression of its target genes *IAA29* and *YUC8*, indicating the possibility of a decreased PIF4 activity (Figure 1E, S2B). Collectively, while our data consolidate the known growth inhibitory role of *ELF3* in PhyB mediated hypocotyl elongation, we propose a novel function of *ELF3* as a growth enhancer under BB. The underlying molecular mechanism of ELF3 mediated growth promotion remains unknown. However, based on its known transcription/activity repressor function (Nieto et al., 2014; Ezer et al., 2017), it seems likely that ELF3 inhibits the function of a growth repressor under BB. If so, CRY1 would represent an attractive candidate. In support of this hypothesis, CRY1 and PIF4 have been shown to physically interact and bind to the same promoter regions (Ma et al., 2015; Pedmale et al., 2015). This

binding decreases PIF4 transcriptional activity in a blue light dependent manner (Ma et al., 2015), which could be explained by a competitive repressor-of-the-repressor model. In this model, CRY1 represses PIF4 transcriptional activity and ELF3 represses CRY1's ability to inhibit PIF4 activity. De-repression of PIF4 would therefore facilitate activation of PIF4 target genes. This model is in line with the known function of ELF3 as a light signaling inhibitor (McWatters et al., 2000; Kolmos et al., 2011).

The molecular mechanism by which GI controls growth is not fully understood. An elongated hypocotyl of *gi* mutants under red and blue light suggested a repressive role in photoreceptor mediated growth inhibition (Huq et al., 2000; Martin-Tryon et al., 2007). Recent data demonstrated that GI requires PIF4 for growth regulation (de Montaigu et al., 2014; Fornara et al., 2015). Since the EC regulates *PIF4 (Nusinow et al., 2011)* and *ELF4* is epistatic to *GI (Kim et al., 2012)*, a role of *GI* upstream of the EC in growth regulation has been proposed (de Montaigu et al., 2014). However, our data, especially an additive hypocotyl phenotype and increased levels of *PIF4* in *elf3 gi* (Figure 1B-E, 5D), advocate an independent repressive action of GI on *PIF4*.

As growth and developmental phenotypes investigated in this study depend on the circadian clock, we asked whether ELF3's and GI's function in the clock might be able to explain the observed effects. By a "gating" mechanism the clock ensures that maximum growth happens at the correct time of day. In WT, under LD growth rates peak in the early morning coinciding with the maximum expression of *PIF4 (Nozue et al., 2007)*.To coordinate this timing of growth rates, TOC1 and EC components including ELF3 repress growth during the late-evening and night, respectively (Nozue et al., 2007; Box et al., 2014; Zhu et al., 2016). In this study, we demonstrate that *GI* is also essential for clock mediated gating. GI represses growth during mid-day to late afternoon, thereby contributing to restricting growth peaks to the morning, resulting in normal rhythmic growth. Consistently, the loss of day and night time gating response in *elf3 gi* double mutants results in uncontrolled elongation growth (Figure 2D). Based on these observations we propose a model of rhythmic growth incorporating *ELF3* and *GI*. In that model ELF3 and GI gate growth mainly by repressing *PIF4* during the night and late afternoon, respectively, allowing it to accumulate only during the early morning under LD. The morning accumulation of *PIF4* induces its downstream targets that consequently trigger cellular growth (Figure 5E).

The gating properties of the circadian clock are mainly dependent on its ability to synchronize internal cellular mechanisms with the external environment. Although after entrainment the clock maintains the same rhythm in the absence of the external input, in nature these free-running conditions almost never exist. Thus, proper clock responses to consistent external cues during a diurnal cycle are crucial for the synchronization of endogenous and environmental signals. Interestingly, arrhythmic clock genotypes, such as null mutants of the EC members *ELF3*, *ELF4* and *LUX*, as well as overexpressors of *CCA1* and *TOC1,* exhibit a non-functional oscillator under free-running conditions, but they are fully capable of generating robust rhythms under diurnal conditions (Fowler et al., 1999; Makino et al., 2002; Hall et al., 2003; Kolmos et al., 2011; Kim et al., 2012). Even higher order clock mutants including *cca1-1 lhy-11 toc1-2,* which lack the entire central oscillator, can generate rhythms under cycling conditions (Yamashino et al., 2008). The data presented in this study demonstrate that the absence of the two components *ELF3* and *GI* is sufficient to make the oscillator arrhythmic under both free-running and even under diurnal conditions (Figure 4A-E). We demonstrated that ELF3 and GI serve as important *Zeitnehmers* that are essential for clock entertainment. In their absence, the oscillator cannot perceive external timing cues provided by cycling light conditions and thus fails to generate rhythmic oscillation of the downstream endogenous outputs. A closer look at the transcriptional profile of the major core-

oscillator genes and the clock-regulated output genes under diurnal conditions in *elf3 gi* suggests that the entire clock-regulated transcriptome seems arrested (Figure 4A-E). As such, even changes in the environmental conditions during a diurnal cycle had no effect on the oscillator and were unable to release the clock-regulated transcriptome from its arrested state (Figure 5A-D). This should rationally lead to a breakdown of any clock-control output pathway. Consistently, photoperiod-responsive flowering and growth was disrupted in *elf3 gi* (Figure 1A-B). Notably, light regulated processes that are independent of the circadian clock seem to be intact in *elf3 gi*. A continuous inhibition of hypocotyl length under increasing photoperiod (Figure 1B, Tables S1-S2) along with marked differences in growth rate during the light and dark phase in *elf3 gi* support this notion (Figure 2D, Table S3). Collectively, our data demonstrate that *ELF3* and *GI* control the circadian clock Zeitgeber-Zeitnehmer interface, enabling the oscillator to synchronize internal cellular mechanisms to the external environment.

Orthologues of *ELF3* and *GI* have been identified in several higher plants. Both genes have been prime breeding targets in crops for flowering time (Faure et al., 2012; Bendix et al., 2015; Panigrahi and Mishra, 2015; Huang and Nusinow, 2016). The *elf3 gi* double mutants develop rather normally and flower at the same time irrespective of the photoperiod (Figure 1A-B). If similar genetic and functional relationships between *ELF3* and *GI* exist in economically important crops as reported here for Arabidopsis, breeders could develop photoperiod-insensitive varieties lacking *ELF3* and *GI* that would be independent of latitudinal photoperiodicity (Soyk et al., 2016).

Materials and methods:

Plant material

All genotypes used were in Ws-2 genetic background. The *elf3-4* null mutant (Liu et al., 2001) was previously described in (Zagotta et al., 1996; Hicks et al., 2001). The *gi-158* mutant was obtained in an ENU (*N*-ethyl-*N*-nitrosourea) genetic screen and will be explained elsewhere. The *gi-158* is possibly a null mutant that contains a premature stop codon resulting in a truncated protein of 146/1173 amino acids. The flowering time and hypocotyl phenotypes of *gi-158* were very similar to the *gi-11* null mutant (Fowler et al., 1999) (Figure S2A-B). The double mutant *elf3-4 gi-158* was generated by crossing *elf3-4* and *gi-158,* and was confirmed by genotyping (Figure S2C). Marker used for genotyping were: *gi-158*, (forward ACTCATTACAACCGTCCCATCTA, reverse, GCGCATGAACACATAGAAGC (XbaI) *elf3-4* (forward TGCAGATAAAGGAGGGCCTA, reverse, ATGGTCCAGGATGAACCAAA.

Growth conditions

For luciferase assays, seeds were surface-sterilized and plated on MS medium containing 3% sucrose. Following ~3 days stratification at 4°C, seedlings were entrained for 7 days, either under LD, ND, SD cycles (~100µmol $m^{-2}s^{-1}$) with constant temperature of 20°C (LD). The bioluminescence measurement and data analysis was performed as described (Hanano et al., 2008). For hypocotyl assays, seedlings were grown on ATS medium, as described previously (Lincoln et al., 1990). Hypocotyl length was determined for seedlings grown under varying photoperiod for 7 days or under RR or BB (light intensity white fluorescent light, 90 µmol $m^{-2}s^{-1}$; light intensity RR and BB: monochromatic LED, 20 µmol $m^{-2}s^{-1}$). The correct spectrum and intensities of red and blue light was confirmed by a spectrometer (UPRtek® MK350S). Seedlings were imaged, and hypocotyl elongation was measured using the *Rootdetection* program (http://www.labutils.de/rd.html). For flowering time measurement, plants were grown on soil containing a 3:1 mixture of substrate and vermiculite in phytochambers (Johnson) with LD, ND, SD cycle (white fluorescent light: 90 µmol $m^{-2}s^{-1}$, constant 22°C). Flowering time was scored at the time of bolting (1 cm above rosette leaves) as the total number of days to bolt. For all experiments, data loggers were used to monitor the growth conditions.

Infra-red photography for growth rate measurement

Seedlings were grown as described above with the following exception: to facilitate imaging unobstructed in air, seedlings were grown vertically on an agar ledge formed by removing part of agar in a square petri plate. Seeds were placed in small ridges on top of the agar. Imaging was started as soon as the cotyledons emerged from the seed coat. Photographs were taken every 60 minutes for 48 hours in LD cycles (white fluorescent light: 30 µmol $m^{-2}s^{-1}$, constant 20°C). To image growth in day-night cycles we built an infrared imaging platform consisting of a modified camera with IR long pass 830 nm cut filters (Panasonic G5). Illumination was achieved using 880 nm IR backlights (Kingbright BL0106-15-29). Image stacks were analyzed using ImageJ (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij). Data loggers were used to monitor the growth conditions.

Expression Analysis

Total RNA was isolated with NucleoSpin® RNA Plant (Macherey-Nagel) following the manufacturer's protocol from 1-week-old seedlings entrained in 12L:12D (90 µmol $m^{-2}s^{-1}$, constant 20°C). Light intensities for BB were 20 µmol $m^{-2}s^{-1}$. Quantitative RT-PCR, and primer sequences were previously

described (Kolmos et al., 2009) with following modifications: ABsolute Blue qPCR SYBR Green (ThermoFisher®) was used instead of iQ SYBR Green (Biorad). Agilent Mx3005P or AriaMx realtime system (Agilent®) were used instead of BioRad. Data loggers were used to monitor the growth conditions.

Figure Legends:

Figure 1. **Photoperiod-responsive flowering and hypocotyl elongation require functional *ELF3* and *GI*.**

(A) Flowering time of Ws-2, *elf3*, *gi* and *elf3 gi* under LD (16h light: 8 h darkness), ND (12h light: 12h darkness), and SD (8h light: 16h darkness). Flowering time was counted as number of days to 1cm bolt. Error bars represent standard deviation (StD). n≥24. Letters above the bars represent statistically significant differences calculated using one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference) Test, p<0.01. **(B)** Hypocotyl length of Ws-2, *elf3*, *gi* and *elf3 gi* under different photoperiods. Numbers at X-axis represent the length of the light period. For instance '8' typify (8h light: 16h darkness), and 12 (12h light: 12h darkness). DD and LL represent constant darkness and constant light, respectively. Seedling were grown for seven days under the respective photoperiod at constant 20°C. Error bars represent standard deviation. n≥18. Letters above the bars represent statistically significant differences among four genotypes under the specified photoperiod (ANOVA with post-hoc Tukey HSD Test, p<0.01). **(C)** Hypocotyl length of Ws-2, *elf3*, *gi* and *elf3 gi* under constant red (RR) or constant blue (BB) light. Plants were grown for 7 days under monochromatic red or blue light at constant 20°C before the pictures were taken and hypocotyl length was measured. Significance as described in **(B)** calculated separately for RR and BB. Experiment was repeated at least three times with similar results. **(D)** Expression of *PIF4, IAA29* and *YUC8* under constant blue (BB) light. Plants were grown for 7 days under monochromatic blue light at constant 20°C before the samples were harvested. Error bars represent the standard error of the mean (SEM) of three biological replicates. Significance as described above, P<0.05. See also Figure S1, Table S1, and Table S2.

Figure 2. ***ELF3* and *GI* repress growth during night and day respectively.**

(A-D) Growth rate of Ws-2, *elf3*, *gi* and *elf3 gi* under LD. Starting from the third day photographs were taken every one hour using a modified Infra red camera. To measure new growth, the time-lapsed images were imported into ImajeJ and hypocotyl length was measured (please see materials and methods for details). Non-shaded are in the graph represents light period (day), and shaded area represents dark period (night). Error bars represent standard error of the mean (S.E.M.), n≥8. Experiment was repeated at least three times with similar results. See also Table S3.

Figure 3. ***ELF3* and *GI* work independently in circadian clock.**

(A) Free-running profile of *CCR2::LUC* expression in Ws-2, *elf3*, *gi* and *elf3 gi*. The plants were entrained for 7 days under 12h:12h light dark cycles, followed by transfer to LL and measurement of *CCR2::LUC* expression for 5 days. Error bars represent SEM and are shown on every third reading. **(B)** absolute Luminescence values of the *CCR2:LUC* profiles shown in (A). Error bars are SEM, n=48. Significance as described in Figure 1. **(C)** Period and **(D)** R.A.E. estimates after entrainment under different photoperiods. Plants were entrained for 7 days under LD, ND or SD before releasing into the free-running condition of constant light and temperature. The *CCR2:LUC* profiles were monitored for 5 days under free-running and period and R.A.E. was calculated. Error bars are SEM, n=48. Significance as

described in Figure 1. Because of the arrhythmic nature of the *elf3* and *elf3 gi*, these lines were excluded from statistical analysis in **(C)**.

Figure 4. ***ELF3* and *GI* are required for clock entrainment.**

(A-E) Transcript accumulation of different circadian clock genes *CCA1* **(A)**, *TOC1* **(B),** *PRR9* **(C)**, *GI* **(D)** and *ELF3* **(E)** in Ws-2, *elf3*, *gi* and *elf3 gi* (*elf3 gi*) under ND (12h light: 12h darkness). Error bars represent the standard deviation of three technical repeats. Expression levels were normalized for *PROTEIN 19 PHOSPHATASE 2a subunit A3* (*PP2A*). Experiment was repeated with similar results. Open bars in the graph represent time in LL, and closed bar represents time in DD.

Figure 5. Endogenous and environmental signals synchronization require functional *ELF3* and *GI*.

(A-D) Transcript accumulation of flowering time genes *GI* **(A)**, *CO* **(B)** and *FT* **(C)** and major growth promoter *PIF4* **(D)** in Ws-2, *elf3*, *gi* and *elf3 gi* (*elf3 gi*) under ND (12h light: 12h darkness). Error bars represent the standard deviation of three technical repeats. Expression levels were normalized for *PROTEIN 19 PHOSPHATASE 2a subunit A3* (*PP2A*). Experiment was repeated with similar results. Open bars in the graph represent time in LL, and closed bar represents time in DD. **(E)** A model of hypocotyl growth. ELF3 and GI repress growth during the night and late-day, respectively by repressing the expression of *PIF4.*

Figure S1.

(A-B) Hypocotyl length of Ws-2, *elf3*, *gi* and *elf3 gi* under different photoperiods as shown in Figure 1B . For clarification, data is split into two photoperiod ranges **(A)** 0-12 and **(B)** 12-24. Growth condition, error bars and statistical analysis as described in Figure 1B. **(C-D)** Hypocotyl length of Ws-2, *elf3*, *gi* and *elf3 gi* under constant white light (LL) , LD, SD and ND. Significance as described in Figure 1. **(E-F)** Hypocotyl growth under monochromatic red or blue light with LD or SD photocycles. Plants were grown for 7 days at constant 20°C.

Figure S2. ***gi-158* is a null mutant.**

(A) Flowering time of Ws-2, *gi-158* and *gi-11* under LD (16h light: 8 h darkness), 1212 (12h light: 12h darkness), and SD (8h light: 16h darkness). Flowering time was counted as number of days to 1cm bolt. Error bars represent standard deviation, n≥24. **(B)** Hypocotyl length of Ws-2, *gi-158* and *gi-11* under ND. Significance as described in Figure 1 within a specific photoperiod. **(C)** confirmation of the *elf3 gi* mutant by genotyping. Two independent double mutant lines were obtained after crossing: *elf3 gi (1) and elf 3 gi (2)*. After genotypic and phenotypic confirmation, only one line *elf3 gi (1)* was used for further experiments.

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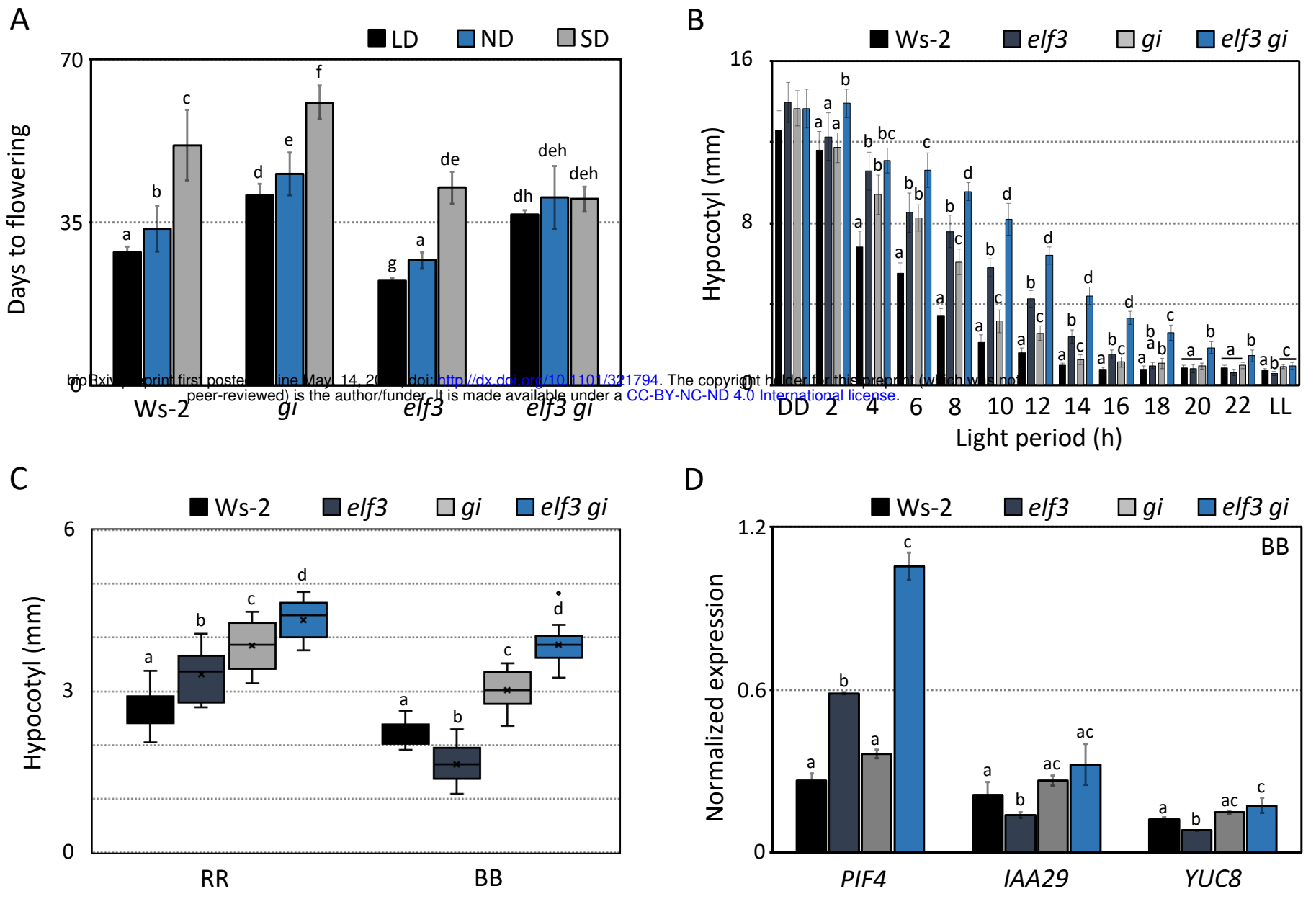


Figure 1. **Photoperiod-responsive flowering and hypocotyl elongation require functional *ELF3* and *GI*.**
(A) Flowering time of Ws-2, *elf3*, *gi* and *elf3 gi* under LD (16h light: 8 h darkness), ND (12h light: 12h darkness), and SD (8h light: 16h darkness). Flowering time was counted as number of days to 1cm bolt. Error bars represent standard deviation (StD). n≥24. Letters above the bars represent statistically significant differences calculated using one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference) Test, p<0.01. **(B)** Hypocotyl length of Ws-2, *elf3*, *gi* and *elf3 gi* under different photoperiods. Numbers at X-axis represent the length of the light period. For instance '8' typify (8h light: 16h darkness), and 12 (12h light: 12h darkness). DD and LL represent constant darkness and constant light, respectively. Seedling were grown for seven days under the respective photoperiod at constant 20°C. Error bars represent standard deviation. n≥18. Letters above the bars represent statistically significant differences among four genotypes under the specified photoperiod (ANOVA with post-hoc Tukey HSD Test, p<0.01). **(C)** Hypocotyl length of Ws-2, *elf3*, *gi* and *elf3 gi* under constant red (RR) or constant blue (BB) light. Plants were grown for 7 days under monochromatic red or blue light at constant 20°C before the pictures were taken and hypocotyl length was measured. Significance as described in **(B)** calculated separately for RR and BB. Experiment was repeated at least three times with similar results. **(D)** Expression of *PIF4, IAA29* and *YUC8* under constant blue (BB) light. Plants were grown for 7 days under monochromatic blue light at constant 20°C before the samples were harvested. Error bars represent the standard error of the mean (SEM) of three biological replicates. Significance as described above, P<0.05.

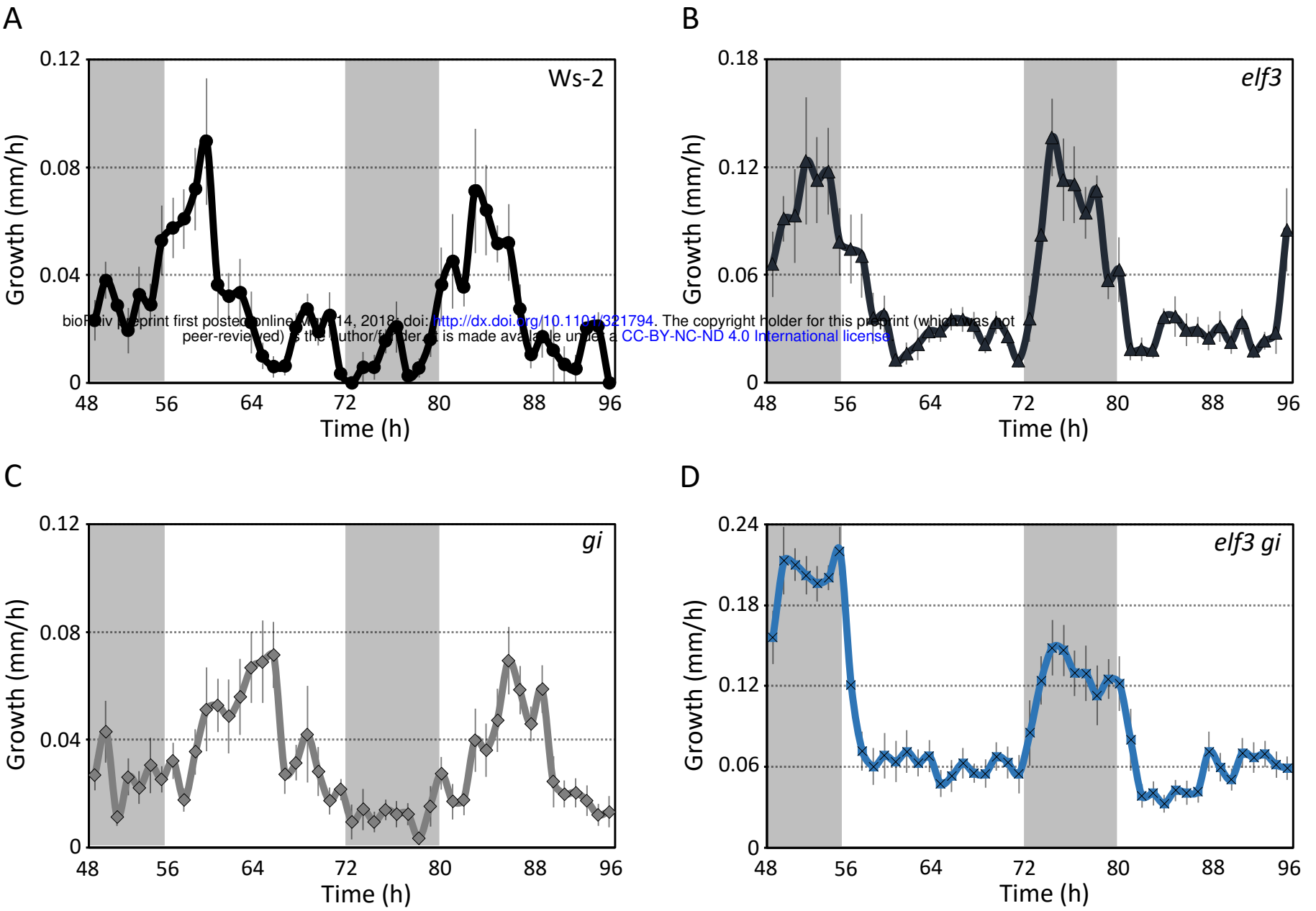


Figure 2. ***ELF3* and *GI* repress growth during night and day respectively.**
(A-D) Growth rate of Ws-2, *elf3*, *gi* and *elf3 gi* under LD. Starting from the third day photographs were taken every one hour using a modified Infra red camera. To measure new growth, the time-lapsed images were imported into ImajeJ and hypocotyl length was measured (please see materials and methods for details). Non-shaded are in the graph represent light period (day), and shaded area represents dark period (night). Error bars represent standard error of the mean (S.E.M.), n≥8. Experiment was repeated at least three times with similar results.

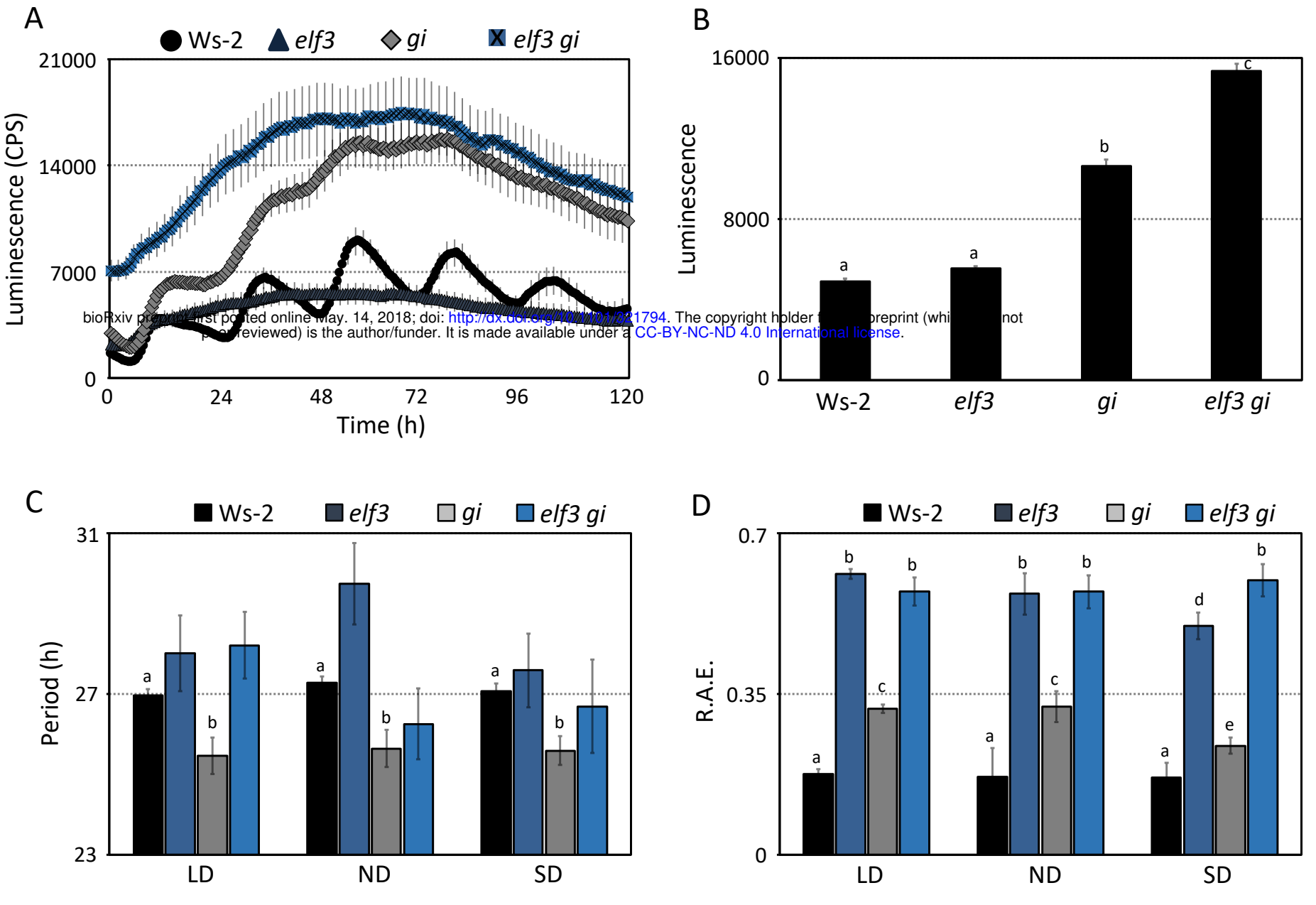


Figure 3. ***ELF3* and *GI* work independently in circadian clock.**
(A) Free-running profile of *CCR2::LUC* expression in Ws-2, *elf3*, *gi* and *elf3 gi*. The plants were entrained for 7 days under 12h:12h light dark cycles, followed by transfer to LL and measurement of *CCR2::LUC* expression for 5 days. Error bars represent SEM and are shown on every third reading. **(B)** absolute Luminescence values of the *CCR2:LUC* profiles shown in (A). Error bars are SEM, n=48. Significance as described in Figure 1. **(C)** Period and **(D)** R.A.E. estimates after entrainment under different photoperiods. Plants were entrained for 7 days under LD, ND or SD before releasing into the free-running condition of constant light and temperature. The *CCR2:LUC* profiles were monitored for 5 days under free-running and period and R.A.E. was calculated. Error bars are SEM, n=48. Significance as described in Figure 1. Because of the arrhythmic nature of the *elf3* and *elf3 gi*, these lines were excluded from statistical analysis in **(C)**.

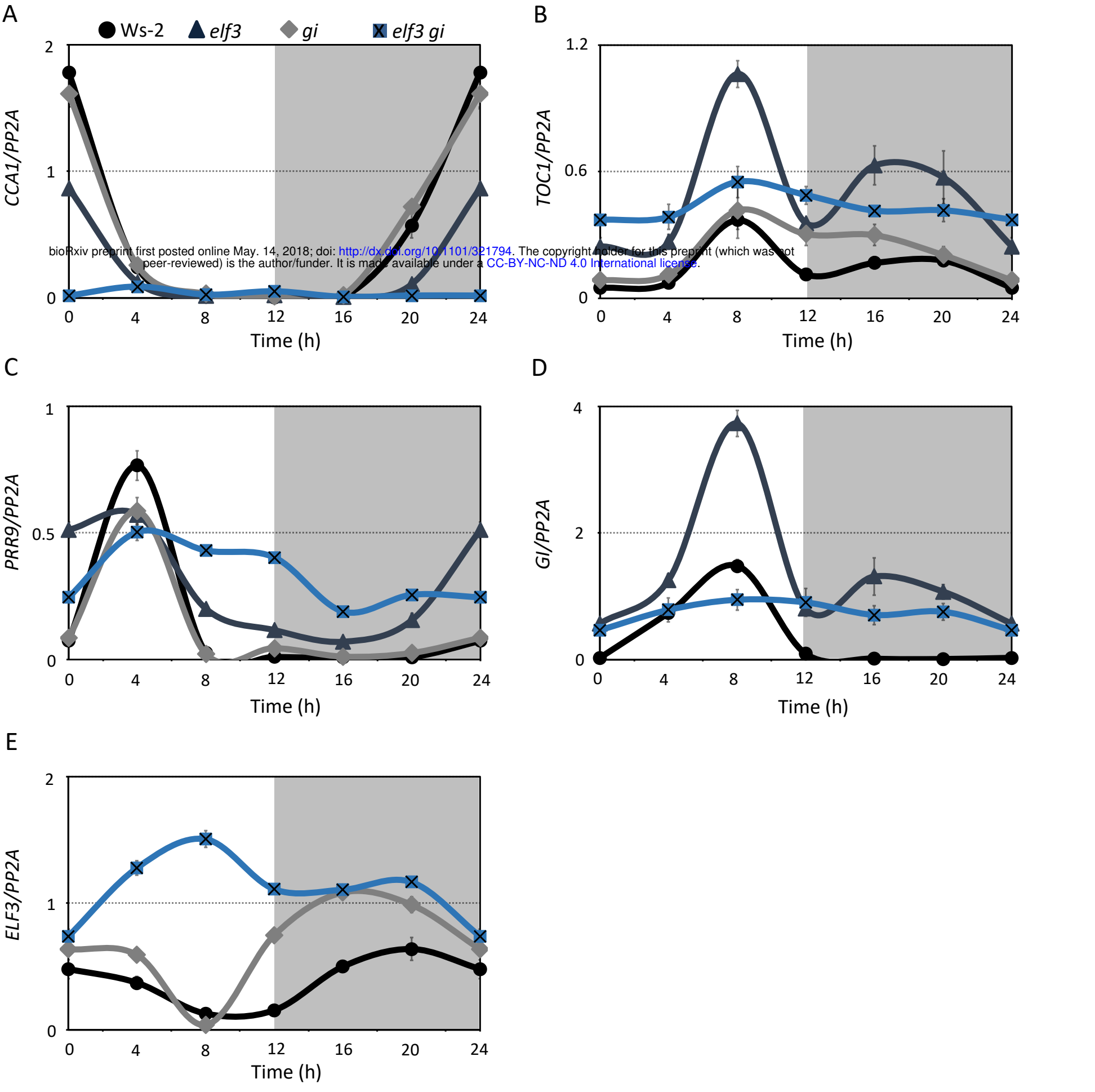
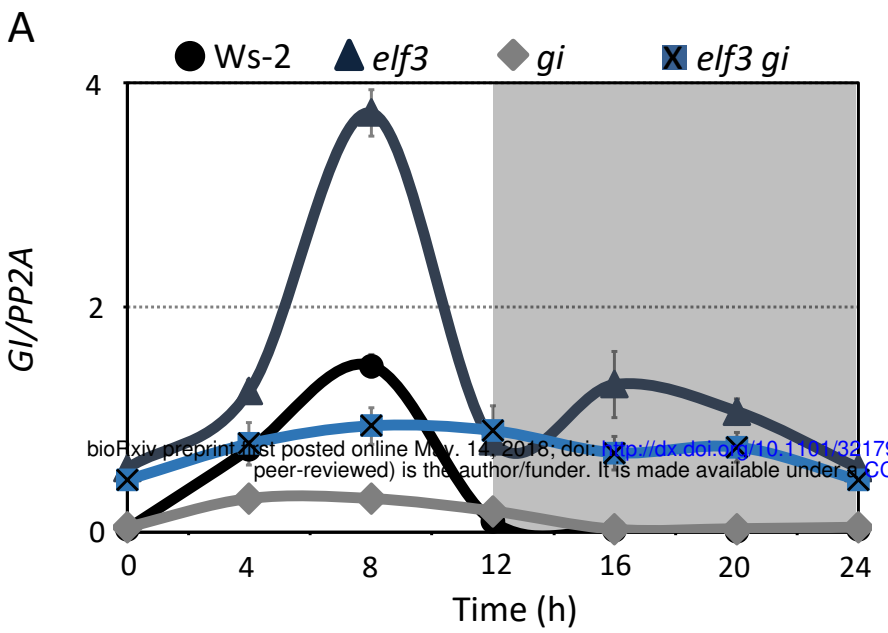
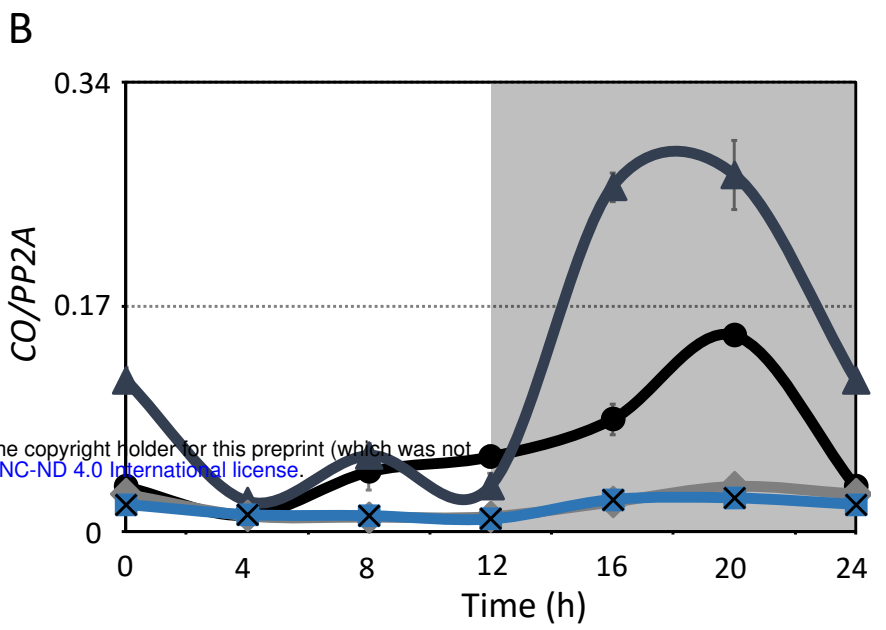
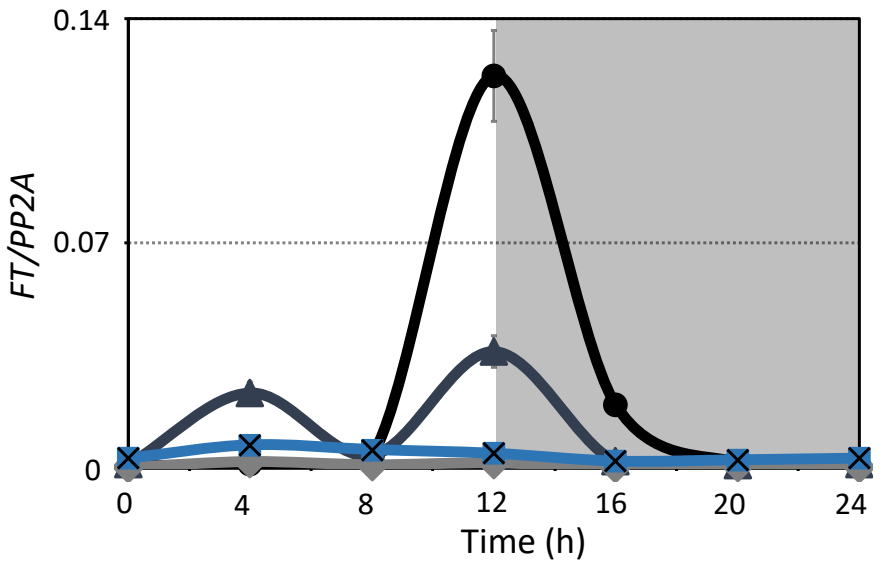
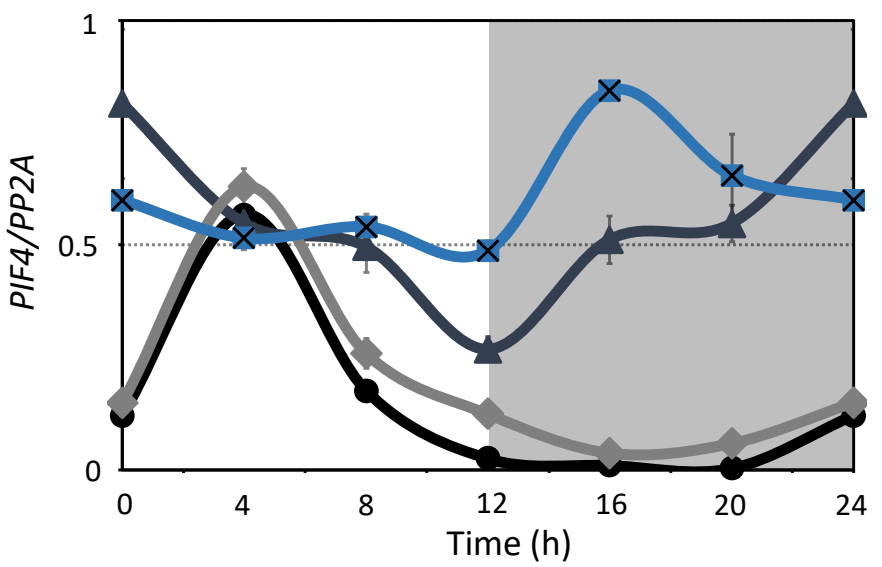


Figure 4. ***ELF3* and *GI* are required for clock entrainment.**
(A-E) Transcript accumulation of different circadian clock genes *CCA1* **(A)**, *TOC1* **(B),** *PRR9* **(C)**, *GI* **(D)** and *ELF3* **(E)** in Ws-2, *elf3*, *gi* and *elf3 gi* (*elf3 gi*) under ND (12h light: 12h darkness). Error bars represent the standard deviation of three technical repeats. Expression levels were normalized for *PROTEIN 19 PHOSPHATASE 2a subunit A3* (*PP2A*). Experiment was repeated with similar results. Open bars in the graph represent time in LL, and closed bar represents time in DD.









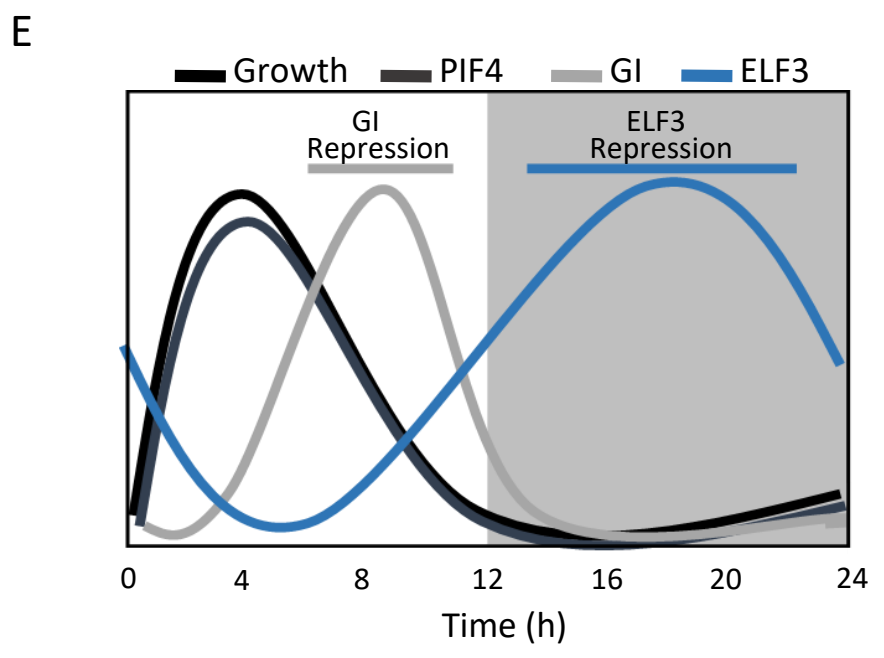


Figure 5. **Endogenous and environmental signals synchronization requires functional *ELF3* and *GI*.**
(A-D) Transcript accumulation of flowering time genes *GI* **(A)**, *CO* **(B)** and *FT* **(C)** and major growth promoter *PIF4* **(D)** in Ws-2, *elf3*, *gi* and *elf3 gi* (*elf3 gi*) under ND (12h light: 12h darkness). Error bars represent the standard deviation of three technical repeats. Expression levels were normalized for *PROTEIN 19 PHOSPHATASE 2a subunit A3* (*PP2A*). Experiment was repeated with similar results. Open bars in the graph represent time in LL, and closed bar represents time in DD. **(E)** A model of hypocotyl growth. ELF3 and GI repress growth during the night and late-day, respectively by repressing the expression of *PIF4.*

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